

***Gerhardtia foliicola*, a new record for Pakistan**

AIMAN IZHAR*, MUHAMMAD USMAN,
MUNAZZA KIRAN, ABDUL NASIR KHALID

*Fungal Biology and Systematics Research Laboratory, Institute of Botany,
University of the Punjab, Quaid-e-Azam Campus-54590, Lahore, Pakistan*

* CORRESPONDENCE TO: aimanizhar25@gmail.com

ABSTRACT—*Gerhardtia foliicola* is reported as a new record for Pakistan. The species is characterized by a reddish brown and hygrophanous pileus, densely crowded lamellae, small sized ellipsoid basidiospores with irregular outline and occurrence on leaf litter and humus in broadleaf forests. Our phylogenetic analysis based on nuclear ribosomal internal transcribed spacer (nrITS) region clustered the Pakistani collections in the same clade with *G. foliicola* sequences from Japan. Detailed description of macro- and micro-characters, habitat, general distribution, and diagnostic features important for delimiting *G. foliicola* from closely related taxa are presented.

KEY WORDS—*Agaricales*, biodiversity, molecular phylogeny, *Lyophyllaceae*, taxonomy

Introduction

Gerhardt (1982) published *Lyophyllum* subg. *Lyophylloopsis* Ew. Gerhardt [non *Lyophylloopsis* Sathe & J.T. Daniel (Sathe & al. 1981)], with *L. incarnatobrunneum* Ew. Bernhardt as its only species. Bon (1994) raised this *subgenus* to generic rank as *Bernhardtia* Bon stat. & nom. nov. (*Agaricales*, *Lyophyllaceae*). The type species, *G. incarnatobrunnea* (Ew. Bernhardt) Bon, is a more recent heterotypic synonym of *G. borealis* (Fr.) Contu & A. Ortega (Contu & Ortega 2002). Distinguishing characters of the genus include siderophilous granulation of basidia, minutely verrucose ornamented basidiospores, pileipellis in form of a cutis or a trichoderm, and the absence of clamp connections (Bon 1994). Vizzini &

al. (2015) emended the genus recently to emphasize smooth or verruculose basidiospores, an absence of clamp connections, and a pileipellis in form of a cutis, trichoderm, or hymeniderm.

Gerhardtia is a small genus (He & al. 2019) with c. seven accepted species which are distributed in Europe, New Zealand, North and South America, and China (Baroni 1981; Bon 1994; Contu & Ortega 2002; Contu & Consiglio 2004; Kalamees 2008; Mešić & Tkalčec 2009; Cooper 2014; Vizzini & al. 2015, 2017; Li & al. 2017; Matheny & al. 2017).

Gerhardtia foliicola, originally described from Japan as *Tricholoma foliicola* Har. Takah. (Takahashi 2001), is characterized by a saprotrophic habit and smooth basidiospores with an irregular outline (Endo & al. 2019). Species of *Tricholoma* (Fr.) Staude are characterized by tricholomatoid stature, smooth, white, and sub-globose to oblong basidiospores, and an ectomycorrhizal association with woody plants (Singer 1986), which conflict with the key characteristics of *Gerhardtia*.

Knowledge about *Gerhardtia* is limited, and previously no species of the genus has been reported from Pakistan. During various mycological expeditions, basidiomata of *G. foliicola* were collected that are presented here as a new species record for Pakistan mycobiota.

Materials & methods

Collection site

Specimens were collected from Kumrat valley which lies at the end of Dir Upper district, Khyber Pakhtunkhwa, Pakistan. The area falls in the alpine zone of the foothills of Hindukush where elevation ranges from 2439 to 3048 m above sea level and the annual rainfall is 52.43 cm (Stucki & Khan 1999, Siddiqui & al. 2013). The area has a dry temperate climate and is dominated by *Pinus wallichiana* A.B. Jacks. and *Cedrus deodara* (Roxb. ex D. Don) G. Don (Gillett 2001, Ahmad & Nizami 2015).

Morphological examination

Basidiomata were collected during July–August 2019 and photographed and morphologically characterized in the field. Munsell (1975) is followed for color designations. For long term preservation, specimens were dried using an electric fan heater. Basidiomata were hand-sectioned and mounted in 5% KOH and in Congo red for contrast purposes. Melzer's reagent was used to check basidiospore amyloidity. Specimens were examined microscopically using a Meiji MX4300H compound microscope. Basidiospore, basidial, basidiole, and pileus and stipe hyphae measurements were determined from at least 20 observations at 1000 × magnification.

The abbreviation [n/l/p] refers to n (number of basidiospores) measured from l (number of basidiomata) and p (number of collections). Basidiospore dimensions are given as (a-)b-c(-d). The abbreviations used are Q = length/width ratio of spores and avQ = average Q from all basidiospores $\pm$ standard deviation (Ge & al. 2010). All specimens were deposited in the Herbarium, Institute of Botany, University of the Punjab, Lahore, Pakistan (LAH).

DNA extraction, PCR and DNA sequencing

Genomic DNA was extracted from 5–20 mg of dried specimens using a modified CTAB method (Bruns 1995). The ITS1 +5.8S ITS2 regions were amplified in a polymerase chain reaction nuclear ribosomal DNA using primers ITS1F and ITS4 (White & al. 1990, Gardes & Bruns 1993). Amplified products were sent to TsingKe China for further purification and bidirectional sequencing.

Phylogenetic analyses

Both forward and reverse primer reads were manually edited and assembled to generate a consensus sequence in BioEdit 7.2.5. (Hall 1999). Homology searches were carried out at NCBI webpage using the BLAST tool, and sequences with closest identity were downloaded. Closest relatives of the species and some other sequences representing the *Lyophyllaceae* were selected following phylogenetic studies (Moncalvo & al. 2000; Hofstetter & al. 2002; Cooper 2014; Bellanger & al. 2015; Vizzini & al. 2015, 2017; Latha & al. 2016; Li & al. 2017; Matheny & al. 2017; Yang & al. 2018; Endo & al. 2019). All ambiguous portions with missing data and gaps were omitted from the final matrix. Online MUSCLE tool by EMBL-EBI, (<http://www.ebi.ac.uk/Tools/msa/muscle/>) was used for multiple sequences alignment (Edgar 2004). The maximum likelihood tree was generated using CIPRES Science Gateway employing RaxML -HPC 2 on XSEDE with 1000 bootstrap pseudoreplicates (Miller & al. 2010). Rapid bootstrap search was performed and GTRGAMMA model was chosen. The generated tree was rooted with *Myochromella boudieri* (Kühner & Romagn.) V. Hofst. & al. (GenBank ITS: AF357047) from Switzerland as outgroup (Matheny & al. 2017). Bootstrap values $\geq 50\%$ were cited on tree as significant.

Phylogenetic results

Our sequences showed 99% identity with *Gerhardtia borealis* (KR673544) from Korea and 99% similarity with *G. foliicola* (LC458831, LC458827, LC458828, LC458834, LC458829, LC458833) from Japan (FIG. 1). A total of 39 ITS sequences were assembled for phylogenetic reconstruction. The final alignment was 732 bp, of which 317 sites were conserved, 372 were variable, 261 were parsimony informative, and 102 were singletons. Maximum likelihood analysis clustered the sequences from our Pakistani collection with Japanese *G. foliicola* with a strong bootstrap support (94%).

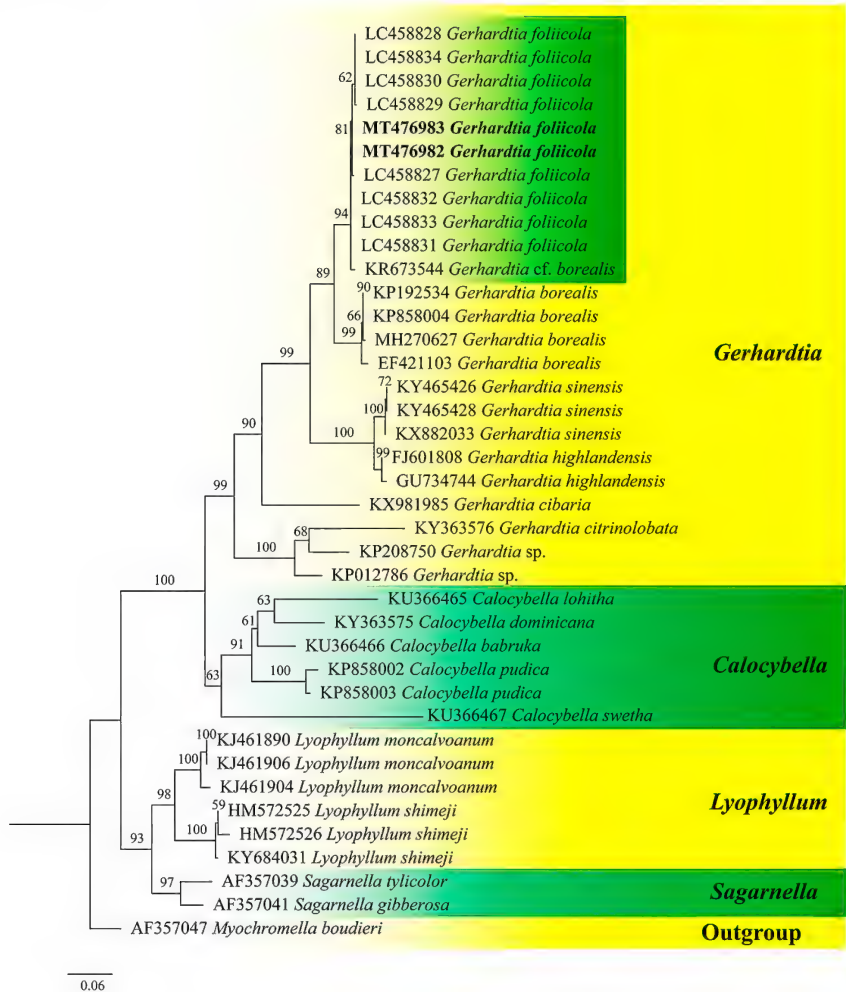


FIG. 1. Maximum Likelihood analyses of *Gerhardtia foliicola* and related taxa based on ITS sequences. The bootstrap values $\geq 50\%$ given above branches. Species collected from Pakistan are highlighted in bold. The analysis included 37 nucleotide sequences.

Taxonomy

Gerhardtia foliicola (Har. Takah.) N. Endo, S. Ushijima, Nagas.,
Sotome, Nakagiri, & N. Maek., Mycoscience 60(6): 324. 2019. FIGS 2, 3

BASIDIOMATA thick, dry, clustered. PILEUS 35–75 mm, hemispherical with rolled margins at first, later becoming convex to applanate, surface

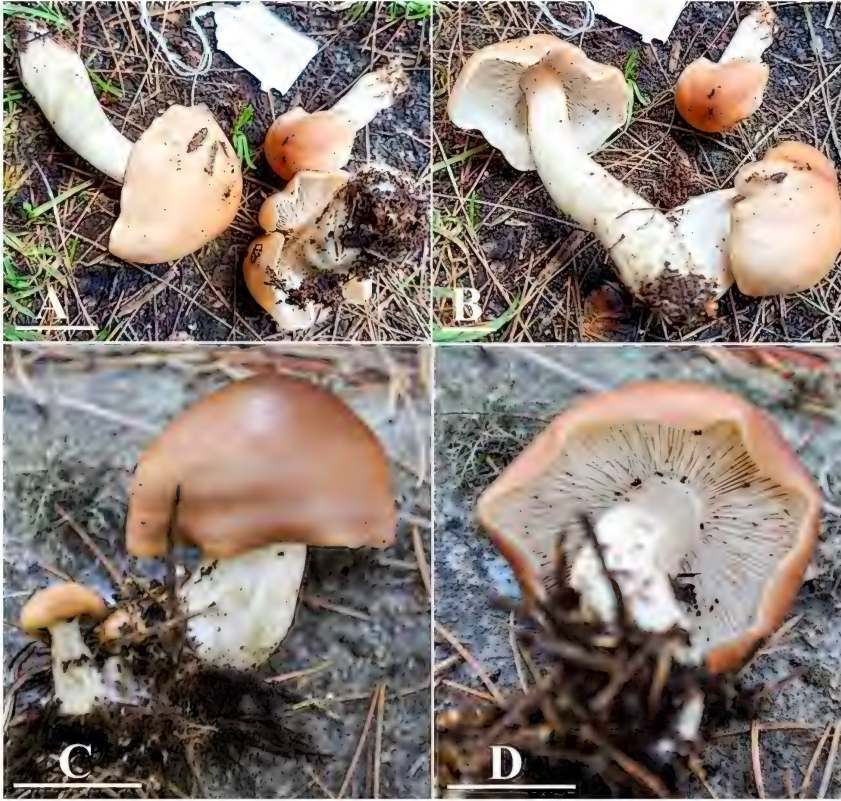


FIG. 2. *Gerhardtia foliicola*: basidiomata.
A, B = LAH 36419; C, D = LAH 36420. Scale bars = 2 cm.

glabrous, shiny when young, later dry, hygrophanous, bright reddish brown (5YR5/6), dull reddish brown (5YR5/4) to dull orange (7.5YR7/4), somewhat paler towards the margin. LAMELLAE white (N10), adnate to emarginate, thin, dense, lamellulae abundant. STIPE 40–120 × 10–30 mm, equal to clavate, sometimes with a bulbous base, slightly fibrillose, base covered with white tomentum, thick, solid, white (N10), light grey (5Y8/2) near the apex. ANNULUS and VOLVA absent. ODOR and TASTE not recorded.

BASIDIOSPORES [40/2/1], (4–)4.7–5.2(–5.5) × (1.7–)1.9–2.5(–2.6) μm , $\text{avl} \times \text{avw} = 4.6 \times 2 \mu\text{m}$, $Q = 2.1\text{--}2.3$, $\text{av}Q = 2.2 \mu\text{m}$, oblong to ellipsoid, smooth, sometimes with tubercles of irregular outline, thin-walled, hyaline in KOH, inamyloid. BASIDIA (19–)20–25(–27) × (4–)4.6–6.2(–7.6) μm , $\text{avl} \times \text{avw} =$

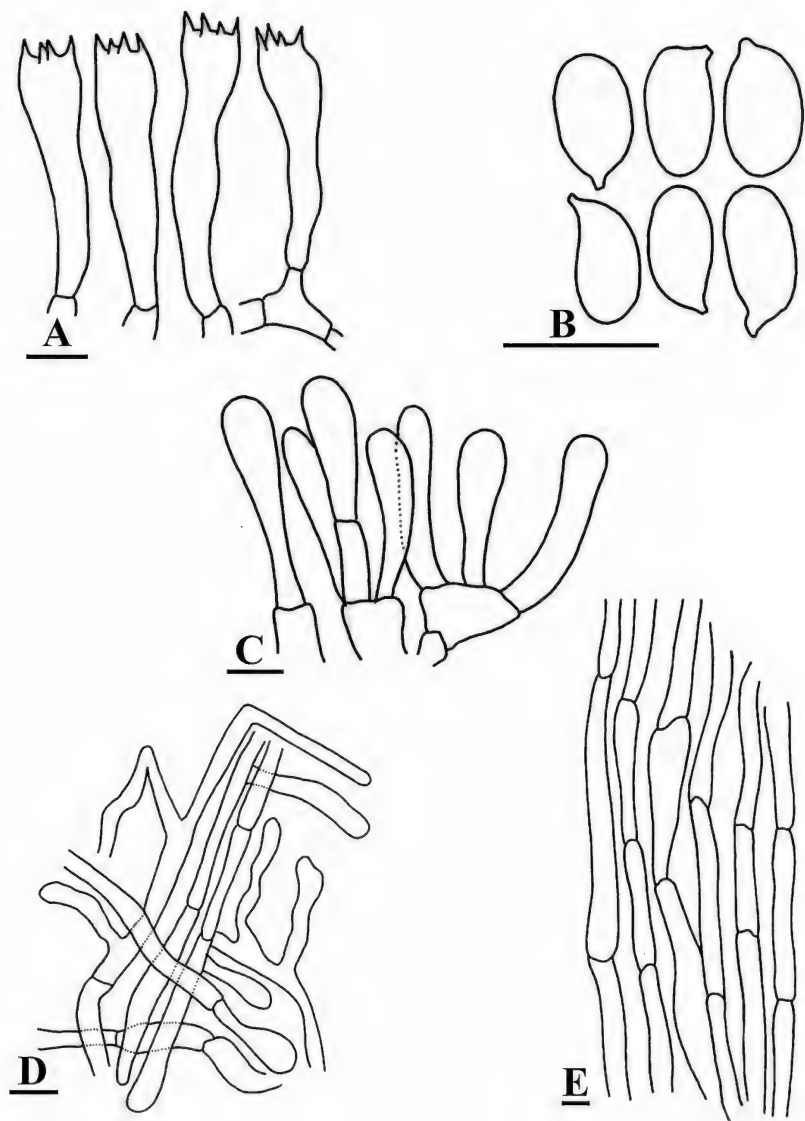


FIG. 3. *Gerhardtia foliicola* (LAH 36419, LAH 36420).
A. Basidia; B. Basidiospores; C. Basidioles; D. Pileus hyphae; E: Stipe hyphae.
Scale bars = 5 μ m. Line drawings by: Aiman Izhar.

22.6 × 5.3 µm, clavate, 4-spored, hyaline in KOH, clamp connections absent. BASIDIOLES (10.6–)13–23(–28) × (2.8–)2.9–4.7(–5) µm, avl × avw = 17.7 × 4.2 µm, clavate, basal cells septate, hyaline in KOH, clamp connections absent. CHEILOCYSTIDIA and PLEUROCYSTIDIA absent. PILEIPELLIS a cutis, hyphae 2–6.5 µm (avw = 4 µm) diam., thin-walled, septate, branched, ends clavate, cylindrical, hyaline in KOH. STIPITPELLIS a cutis of parallel hyphae, 4–11 µm (avw = 7.7 µm) diam., hyaline in KOH, frequently septate, clamp connections absent.

HABITAT & DISTRIBUTION— Growing on leaf litter under cedars and pines. This species was originally reported from Japan and now for the first time from Khyber Pakhtunkhwa province, Pakistan.

SPECIMENS EXAMINED—**PAKISTAN. KHYBER PAKHTUNKHWA:** Dir (Upper) district, Kumrat valley, 35°34'56"N 72°10'10"E, elev. 2439 m, found among pine needles, 25 July 2019, Abdul Nasir Khalid K-204 (LAH 36419; GenBank: MT476982; found on forest floor under *Cedrus deodara* and *Pinus wallichiana*, 15 August 2019, Abdul Nasir Khalid KU-19 (LAH36420; GenBank: MT476983).

Discussion

Gerhardtia foliicola, originally described from Japan as *Tricholoma foliicola*, is characterized by its habit on leaf litter, a dry, glabrous and hygrophanous, reddish brown pileus, adnate and very crowded lamellae, small ellipsoid basidiospores, and hyphae lacking clamp connections (Takahashi 2001). After taxonomic reconsideration, Reschke & al. (2018) recommended transfer of *T. foliicola* to *Gerhardtia*, a recombination later supported by molecular phylogenetic analyses (Endo & al. 2019). Takahashi (2001) reported this species from a broadleaf forest dominated by *Quercus myrsinifolia* and *Q. serrata*; Endo & al. (2019) reported it as growing on humus or on leaf or bark litter of coniferous or broadleaf trees. Our findings confirm its occurrence in coniferous forests.

Our molecular phylogenetic analysis clustered the Pakistani collections in the same clade with Japanese collections of *Gerhardtia foliicola* and with a specimen from South Korea (Ulleungdo) labeled as *G. cf. borealis* (KR673544), which probably also represents *G. foliicola*. Phylogenetically our nrITS sequences have a single nucleotide difference at the 395 bp position when compared with the Japanese sequences. The Pakistani *G. foliicola* specimens possess a larger pileus, longer stipe, oblong to ellipsoid basidiospores, and thinner pileus hyphae contrasting slightly with the Japanese collections (characterized by a smaller pileus, shorter stipe, sub-cylindrical, ellipsoid or subfusiform basidiospores, and broader pileipellis hyphae). Geographically,

Japanese collections are recorded from temperate region and on humus and litter under conifers (*Pinus thunbergii* Parl., *P. densiflora* Siebold & Zucc., *P. parviflora* Siebold & Zucc., *Tsuga diversifolia* (Maxim.) Mast., and broadleaf trees (*Quercus* spp.), (Endo & al. 2019). The Pakistani specimens were recorded from a dry temperate climate under *Cedrus deodara* and *Pinus wallichiana*.

Gerhardtia borealis, a sister species to *G. foliicola* is morphologically the closest counterpart. However, it differs from *G. foliicola* by its quite distant lamellae, larger ($6\text{--}8.5 \times 4\text{--}5\text{ }\mu\text{m}$) basidiospores, and growth on clay or sandy soil in coniferous forests (Contu & Consiglio 2004). This species is also phylogenetically well separated, forming a monophyletic clade that is sister to *G. foliicola* (Gerhardt 1982, Bigelow 1985, Contu & Consiglio 2004).

Acknowledgements

Higher Education Commission, Pakistan is thanked for financial assistance under National Research Program for Universities, project no. 20-3383/NRPU/R&D/HEC/14/184. Authors are grateful to Dr. Junaid Khan (Center for Plant Sciences and Biodiversity, University of Swat, Pakistan), Dr. Ting Li (Guangdong Institute of Microbiology, Guangzhou, China), and Dr. Armin Mešić (Ruđer Bošković Institute, Zagreb, Croatia) for their peer reviews and helpful comments to improve this manuscript.

Literature cited

- Ahmad A, Nizami SM. 2015. Carbon stocks of different land uses in the Kumrat valley, Hindu Kush Region of Pakistan. *Journal of Forestry Research* 26: 57–64. <https://doi.org/10.1007/s11676-014-0008-6>
- Baroni TJ. 1981. A revision of the genus *Rhodocybe* Maire (*Agaricales*). *Beihefte zur Nova Hedwigia* 67. 194 p.
- Bellanger JM, Moreau PA, Corriol G, Bidaud A, Chalange R, Dudova Z, Richard F. 2015. Plunging hands into the mushroom jar: a phylogenetic framework for *Lyophyllaceae* (*Agaricales*, *Basidiomycota*). *Genetica* 143: 169–194. <https://doi.org/10.1007/s10709-015-9823-8>
- Bigelow HE. 1985. North American species of *Clitocybe*, part II. *Beihefte zur Nova Hedwigia* 81: 281–471.
- Bon M. 1994. Deux *Lyophylloideae* interessantes et le genre *Gerhardtia* st. et nom. nov. *Documents Mycologiques* 24: 65–68.
- Bruns TD. 1995. Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. In: *The significance and regulation of soil biodiversity*. Springer Netherlands, pp. 63–73. https://doi.org/10.1007/978-94-011-0479-1_5
- Contu M, Ortega A. 2002. Studi sulle *Lyophyllaceae* della Sardegna. V. Morfologia sporale di *Rugosomyces pudicus* Bon & Contu ed implicazioni sulla sua posizione sistematica. *Boletín de la Sociedad Micológica de Madrid* 26: 171–176.
- Contu M, Consiglio G. 2004. Il genere *Gerhardtia* (*Lyophyllaceae*) in Europa, conosservazioni sulle rimanenti specie conosciute. *Micologia e Vegetazione Mediterranea* 19: 151–162.

- Cooper JA. 2014. New species and combinations of some New Zealand agarics belonging to *Clitopilus*, *Lyophyllum*, *Gerhardtia*, *Clitocybe*, *Hydnangium*, *Mycena*, *Rhodocollybia* and *Gerronema*. *Mycosphere* 2: 263–288. <https://doi.org/10.5943/mycosphere/5/2/2>
- Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5: 113. <https://doi.org/10.1186/1471-2105-5-113>
- Endo N, Ushijima S, Nagasawa E, Sugawara R, Okuda Y, Sotome K, Nakagiri A, Maekawa N. 2019. Taxonomic reconsideration of *Tricholoma foliicola* (*Agaricales*, *Basidiomycota*) based on basidiomata morphology, living culture characteristics, and phylogenetic analyses. *Mycoscience* 60: 323–330. <https://doi.org/10.1016/j.myc.2019.07.002>
- Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118. <https://doi.org/10.1111/j.1365-294x.1993.tb00005.x>
- Ge ZW, Yang ZL, Vellinga EC. 2010. The genus *Macrolepiota* (*Agaricaceae*, *Basidiomycota*) in China. *Fungal Diversity* 45: 81–98.
- Gerhardt E. 1982. Über zwei neue Tricholomataceen: *Collybia hebelomoides* und *Lyophyllum incarnatobrunneum*, gefunden in Berlin. *Zeitschrift für Mykologie* 48: 239–243.
- Gillett S. 2001. Tribesmen, politics, opium and development in Dir, Pakistan. *Asian Affairs* 32: 268–278. <https://doi.org/10.1080/714041446>
- Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.
- He MQ, Zhao RL, Hyde KD, Begerow D, Kemler M, Yurkov A, McKenzie EHC & al. 2019. Notes, outline and divergence times of *Basidiomycota*. *Fungal Diversity* 99: 105–367. <https://doi.org/10.1007/s13225-019-00435-4>
- Hofstetter V, Cléménçon H, Vilgalys R, Moncalvo JM. 2002. Phylogenetic analyses of the *Lyophylleae* (*Agaricales*, *Basidiomycota*) based on nuclear and mitochondrial rDNA sequences. *Mycological Research* 106: 1043–1059. <https://doi.org/10.1017/S095375620200641X>
- Kalamees K. 2008. *Gerhardtia* Bon. 496–497, in: H Knudsen & J Vesterholt (eds). *Funga Nordica*. Agaricoid, boletoid and cyphelloid genera. Nordsvamp, Denmark.
- Latha KPD, Raj KNA, Cherolil T, Sharafudheen SA, Manimohan P. 2016. Three new species of *Calocybella* from India based on morphology and molecular phylogeny. *Phytotaxa* 255: 133–143. <https://doi.org/10.11646/phytotaxa.255.2.2>
- Li T, Li TH, Wang CQ, Deng WQ, Song B. 2017. *Gerhardtia sinensis* (*Agaricales*, *Lyophyllaceae*), a new species and a newly recorded genus for China. *Phytotaxa* 332: 172–180.
- Matheny PB, Baroni TJ, Simoni A, Rojas MEH, Sánchez-García M, Gates GM. 2017. The wild edible mushroom *Pleurocollybia cibaria* from Peru is a species of *Gerhardtia* in the *Lyophyllaceae* (*Agaricales*). *Cryptogamie Mycologie* 38: 205–212. <https://doi.org/10.7872/crym/v38.iss2.2017.205>
- Mešić A, Tkalčec Z. 2009. Studies on Croatian *Basidiomycota* 1: *Gerhardtia piperata* (*Agaricales*). *Mycotaxon* 110: 413–421. <https://doi.org/10.5248/110.413>
- Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Proceedings of the Gateway computing environments workshop (GCE)*. New Orleans, Louisiana, pp. 1–8. <https://doi.org/10.1109/GCE.2010.5676129>
- Moncalvo JM, Lutzoni FM, Rehner SA, Johnson J, Vilgalys R. 2000. Phylogenetic relationship of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic Biology* 49: 278–305. <https://doi.org/10.1093/sysbio/49.2.278>
- Munsell AH. 1975. Soil color charts. Munsell Color Co, Baltimore, MD.

- Reschke K, Popa F, Yang ZL, Kost G. 2018. Diversity and taxonomy of *Tricholoma* species from Yunnan, China, and notes on species from Europe and North America. *Mycologia* 110: 1089–1109. <https://doi.org/10.1080/00275514.2018.1512295>
- Sathe AV, Deshpande S, Kulkarni SM, Daniel JT. 1981 [“1980”]. *Agaricales* (mushrooms) of South-west India – Part I: *Agaricales* (mushrooms) of Maharashtra State. Maharashtra Association for the Cultivation of Science, Monograph 1. 114 p.
- Siddiqui MF, Shaukat SS, Ahmed M, Khan N, Khan IA. 2013. Vegetation environment relationship of conifer dominating forests of moist temperate belt of Himalayan and Hindukush regions of Pakistan. *Pakistan Journal of Botany* 45: 577–592.
- Singer R. 1986. The Agaricales in modern taxonomy (4th ed.). Koenigstein: Koeltz Scientific Books.
- Stucki B, Khan HA. 1999. Working plan for Utror-Desan forests of Kalam Forest Division. Nizam Printing Press, Peshawar.
- Takahashi H. 2001. Notes on new *Agaricales* of Japan 3. *Mycoscience* 42: 355–360. <https://doi.org/10.1007/BF02461218>
- Vizzini A, Consiglio G, Setti L, Ercole E. 2015. *Calocybella*, a new genus for *Rugosomyces pudicus* (*Agaricales*, *Lyophyllaceae*) and emendation of the genus *Gerhardtia*. *IMA Fungus* 6: 1–11. <https://doi.org/10.5598/imafungus.2015.06.01.01>
- Vizzini A, Angelini C, Ercole E. 2017. Is the species diversity in the lyophylloid genera *Calocybella* and *Gerhardtia* (*Agaricales*, *Basidiomycota*) underestimated? Two new species from the Dominican Republic. *Phytotaxa* 291: 241–252. <https://doi.org/10.11646/phytotaxa.291.4.1>
- White TJ, Bruns TD, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in: MA Innis & al. (eds). *PCR Protocols: a guide to methods and applications*. Academic Press, San Diego. <https://doi.org/10.1016/B978-0-12-372180-8.50042-1>
- Yang SD, Huang HY, Zhao J, Zeng NK, Tang LP. 2018. *Ossicaulis yunnanensis* sp. nov. (*Lyophyllaceae*, *Agaricales*) from southwestern China. *Mycoscience* 59: 33–37. <https://doi.org/10.1016/j.myc.2017.07.008>